Pilot Scale Extraction of Proteolytic Enzyme Bromelain from Pineapple (*Ananas comosus*)

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Abstract

Bromelain is a complex of proteolytic enzymes obtained from pineapple (*Ananas comosus*) fruits and stem. These proteolytic enzymes are tools that promise an impressive number of medical and therapeutic uses, particularly as anti-inflammatory agents, dietary supplements and analgesics. The purpose of this study was to develop a pilot scale bromelain extraction process. The bromelain extraction method using pineapple fruit core in this study has yielded a total protein content of 9.33 mg/ml with the proteolytic activity of 1400 GDU/ml. An increase in homogenization cycle to increase enzyme yield has proven to be successful. Triple-loop homogenization has shown three times higher proteolytic activity than non-homogenized sample.

Keywords: Bromelain; homogenized; proteolytic enzyme.

1. Introduction

Bromelain is a mixture of proteolytic enzymes extracted from the fruit and stem of pineapple plant, *Ananas comosus*. Bromelain was initially named for any proteases obtained from various species of *Bromeliaceae* [10]. The major proteinase used in this

study is specifically referred to as fruit bromelain, obtained from the pineapple fruit. This enzyme is also known as juice bromelain, ananase, bromelase, extranase, pinase, pineapple enzyme, traumanase and fruit bromelain FA2. Bromelain also contains small amounts of other physically active compounds, such as comosain, ananain and other unidentified components [11]. The partial structural sequence of bromelain, which showed unique portions of the molecule, such as the amino and carboxyl-termini has been studied [3, 6] and compared with the sequence of the corresponding portions of papain [6]. It was found that the amino acid sequence in bromelain is about 40% homologous with that of papain, around the reactive sulfhydryl group.

Bromelain, the main active component showing proteolytic activity has been first shown to be anti-inflammatory. The other primary uses of these proteolytic enzymes also include dietary supplements as digestive enzymes, pain relievers and inhibition of platlet aggregation [11]. A number of animal as well as human studies have demonstrated the relative safety of orally-administered bromelain. Bromelain has been proven to potentially modify inflammation within the gastrointestinal tract via local proteolytic activity within the colonic microenvironment [8] and cause a dose-dependent decrease of bradykinin levels at the inflammatory site of rats with a kaolin-induced inflammation [13]. A current study also investigated the effects of bromelain on mild acute knee pain, which effectively reduced physical pain and improved general well-being of adults suffering mild knee pain in a dose-dependent manner [1]. Bromelain treatment has also been proven to alter leukocyte expression of cell surface molecules involved in cellular adhesion and activation [7], as well as modulates T cell and B cell immune responses in vitro and in vivo [5].

The available commercial chemical and nutraceutical preparations of bromelain mostly contain stem bromelain. A study of proteinase activity and stability of natural fruit bromelain preparation reflected the composition of proteinase activity of fruit bromelain to be higher than stem bromelain. However, the proteinase stability of stem bromelain is higher than fruit bromelain [9]. Being one of the world producers of pineapple, Malaysia is capable of producing sufficient bromelain for commercialization as pineapple wastes are produced every day. Pineapple wastes like the core parts can be utilized to extract bromelain proteinases. The purpose of this study was to develop a pilot scale preparation

of bromelain from pineapple wastes (pineapple core) using extraction and homogenization process, as well as to determine its total protein content and proteolytic activity.

2. Materials and Methods

2.1 Extraction and homogenization of bromelain from pineapple

The pineapple cores were collected from Pineapple Cannery of Malaysia Sdn. Bhd. at Pekan Nanas Johor. The pineapple cores were initially cut and blended. The crude juice was then divided into three parts and treated separately by extraction with single-loop homogenization at 100 bar, triple-loop homogenization (3×100 bar) and extraction without homogenization. The homogenization process using homogenizer from APV Homogeniser BmbH was carried out to disrupt pineapple cells, decrease juice viscosity and release the intracellular enzyme. The crude bromelain was further centrifuged twice (4°C, 5 min) at 10 000g using refrigerated centrifuge (Sigma 3K20, B.Braun). The clarified pineapple juice of about 1 L was concentrated by precipitating overnight using 55% ammonium sulfate (Fischer Scientific). The precipitated crude bromelain was dissolved in 0.02 M acetate buffer, pH 4.8 and freeze-dried. Figure 1 shows the process flow of bromelain extraction from pineapple with homogenization and non-homogenization. Crude juice, clarified samples, concentrated samples and freeze-dried samples from each extraction process were collected for further analysis.

2.2 Determination of total protein content

The protein content of the collected samples were determined using the bicinchoninic acid (BCA) assay (Pierce Chemical, Rockford, IL) and measured at 562 nm with bovine serum albumin (BSA) as standard.

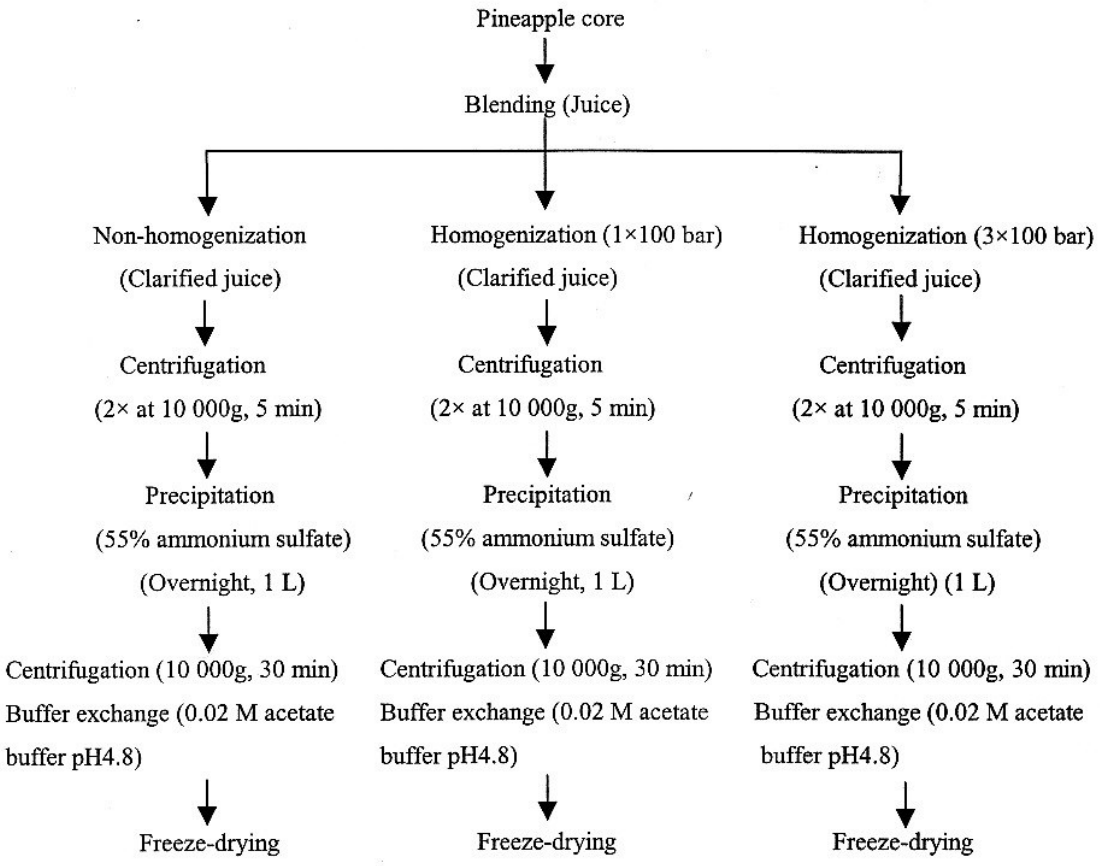

Fig. 1 Process flow of bromelain extraction from pineapple (by non-homogenization, single-loop homogenization and triple-loop homogenization).

2.3 Proteolytic activity measurement

Bromelain proteolytic activity was measured using the gelatin digestion unit analytical method. One gelatin digestion unit is the amount of enzyme which will liberate, after 20 min of digestion at 45°C, 1 mg of amino nitrogen from a standard gelatin solution at pH 4.5. 1-ml of bromelain sample (1.0 mg/ml) was added to 25 ml of gelatin substrate, which had been previously warmed to 45°C for 5 min in a water bath. After 20 min of incubation at 45°C, the mixture was added with 0.1 ml of 3% hydrogen peroxide, swirled and incubated again for 5 min. The mixture was then adjusted to pH 6.0 with 0.1 N NaOH and with constant stirring, 10 ml of 37% of formaldehyde; pH 9.0 was added with the pH recorded after 10 sec and 1 min. The mixture was titrated to pH 9.0 with 0.1

N NaOH and its titration volume was recorded (test titer, T). The digestion was repeated to obtain blank titer, B but with the addition of 0.1 ml of 3% hydrogen peroxide before adding standard bromelain of 50 mg/ml, (Fluka BioChemika). Gelatin digestion unit (GDU) for the above method is defined as

$$GDU/g = \frac{(T-B)\times 14\times N\times 50}{Wt(g)} \quad (1)$$

where T is the test titer (ml), B is the blank titer (ml), N is the normality of standardized NaOH and Wt (g) is the initial weight of enzyme.

3. Results and discussion

3.1 Effect of homogenization on total protein yield and proteolytic activity

The total protein content of bromelain obtained from 3 different processes (without homogenization, single-loop homogenization and triple-loop homogenization) was carried out. The total protein content and proteolytic activity of all the samples tested is varied. The sample that was homogenized three times (triple-loop) showed the highest total protein yield, at 12.13 mg/ml. The amounts of extracted protein in single-loop homogenized and non-homogenized sample are 9.33 mg/ml and 10.4 mg/ml respectively (Figure 2). The protein content in single-loop homogenized bromelain is slightly lower than non-homogenized bromelain. This indicates that one passage/loop of homogenization does not show any significant changes in total protein yield. However, increased number of homogenization passages increased total protein yield. This result shows that high pressure homogenization is capable of disrupting pineapple cells, releasing the intracellular enzyme without denaturation and can be used to increase protein yield. Similar result was detected by Sathivel *et. al* (June,2000) on homogenization study using pineapple fruit and its wastes. It is interesting to see that there is a significant difference in total protein content of crude juice in comparison to the clarified juice as indicated in Figure 2.

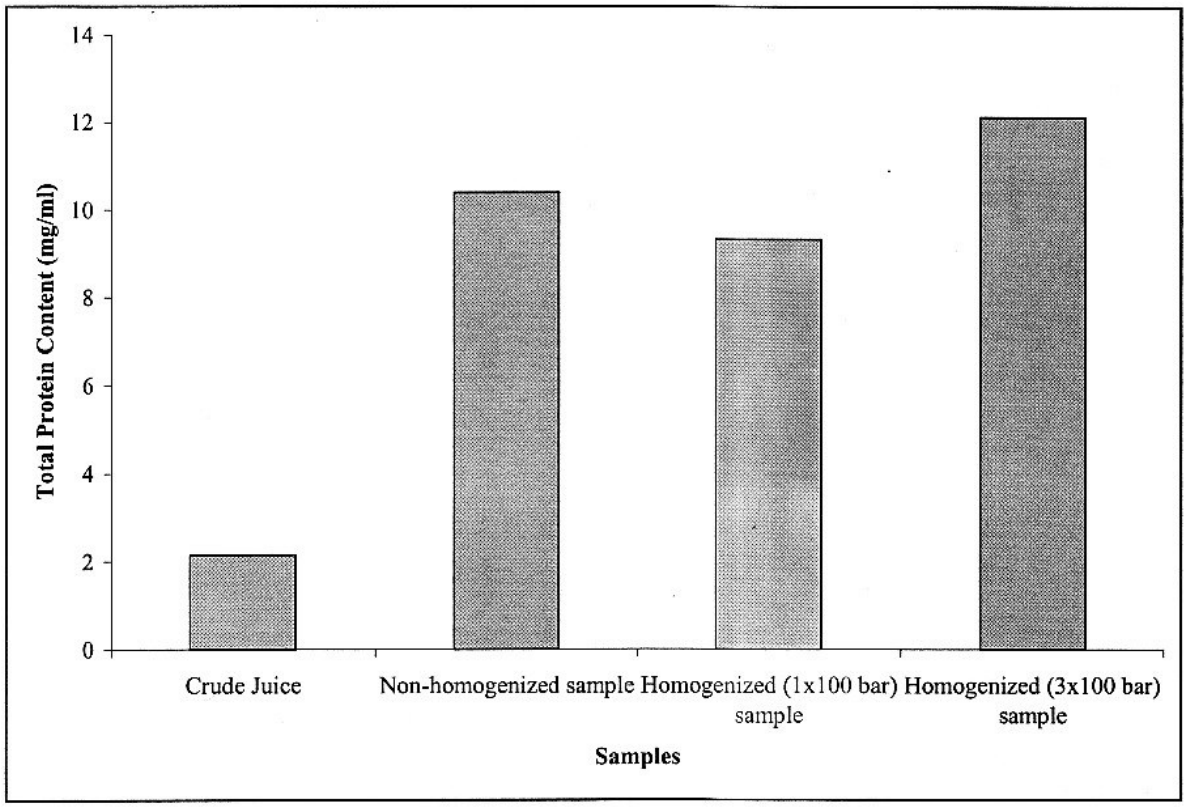

Fig. 2: Determination of total protein content for different samples. The samples were crude juice, non-homogenized sample, homogenized (1×100 bar) sample and homogenized (3×100 bar) sample after freeze-drying process.

Analysis of the enzyme activity using the gelatin digestion unit method determined a total content of active proteinases in the extract preparation. The application of homogenization process resulted in an increase of enzyme activity yield. The bromelain sample that was homogenized 3 times showed the highest proteinase activity at 5100 GDU/g as shown in Figure 3. The difference of enzyme activity between non-homogenized (1600 GDU/g) sample and single-loop homogenized (1400 GDU/g) sample is only 200 GDU/g and is considered negligible in this study. This result is equivalent to the total protein content described previously, where the increased homogenization passages increased the enzyme activity of bromelain. Table 1 shows a summary of protein content and enzyme activity comparison of non-homogenized and homogenized samples.

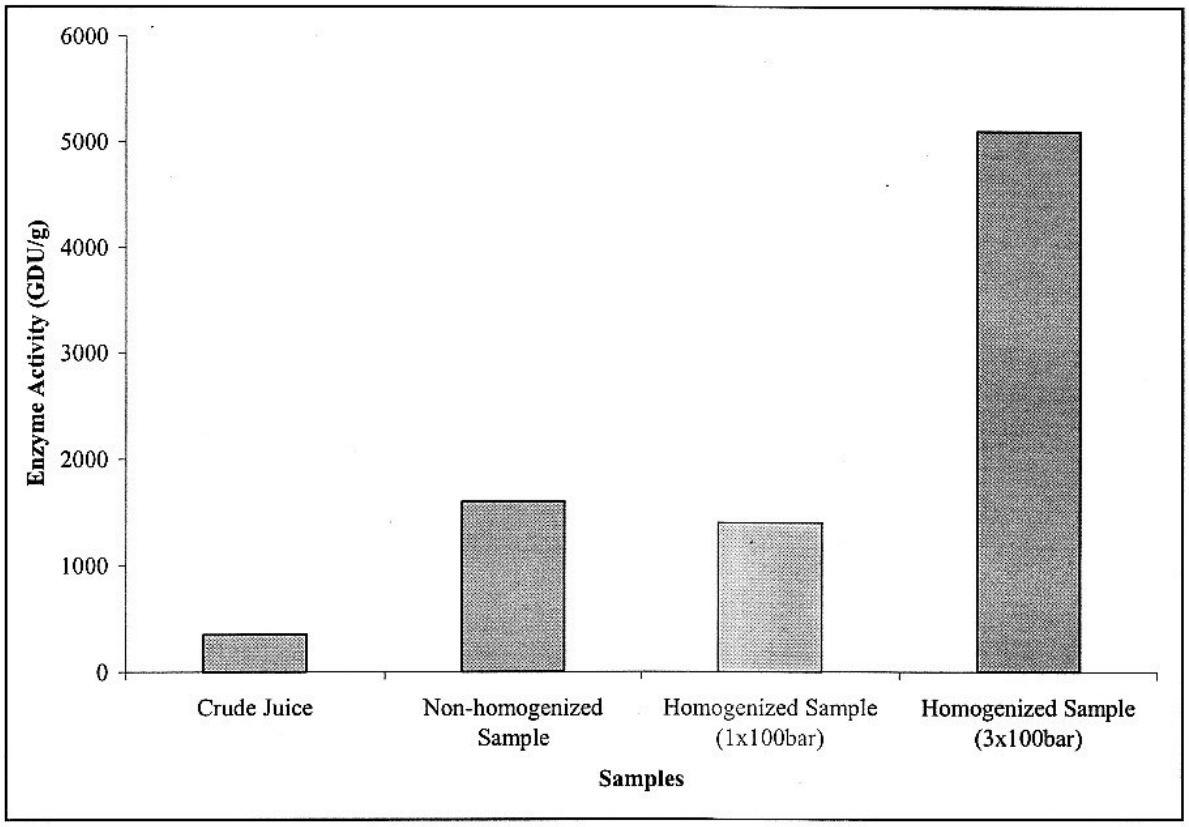

Fig. 3: Comparison of enzyme activities in different bromelain samples. The samples were crude juice, non-homogenized sample, homogenized (1×100 bar) sample and homogenized (3×100 bar) sample after freeze drying process.

Samples	Total Protein Content (mg/ml)	Enzyme Activity (GDU/g)
Crude juice	2.15	350
Non-homogenized sample	10.40	1600
Homogenized sample (1×100 bar)	9.33	1400
Homogenized sample (3×100 bar)	12.13	5100

Table 1: Summary of protein content and enzyme activity comparison of non-homogenized and homogenized samples.

3.2 *Enzyme activity and protein yield at different processing steps*

The enzyme activity and protein yield at different processing steps were determined in this study. This analysis is important to study the potential loss of proteolytic enzymes during the extraction process. Figure 4 and Figure 5 show the total protein content and proteolytic activity of bromelain samples in each processing step during the extraction process (with single-loop homogenization). The purpose of analyzing the protein content and enzyme activity during the process operation was to determine whether each process operation would affect the total protein yield and proteolytic activity. Analysis was carried out using equivalent amount of samples in each processing step. An increase in protein concentration was observed during the whole process. Crude juice has the lowest protein content value at 2.15 mg/ml while the freeze-dried sample showed the highest total protein content of 9.33 mg/ml (Figure 4). A 55% of ammonium sulfate used during sample protein precipitation concentrated and separated larger proteins from small proteins, thus increasing the total protein content of crude bromelain collected.

Freeze-drying process was applied in the processing step to further concentrate the sample as a way for enzyme storage. Although the freeze-dried sample has the highest protein content, the quality of the protein is unknown. Thus, we determined the proteolytic activity of the samples using the gelatin digestion unit analytical method, as described in Materials and Methods. The bromelain activity of the samples which were taken from each processing step of extraction process was analyzed. Single-loop homogenization process showed the highest activity after ammonium sulfate precipitation at 7525 GDU/g (Figure 5). The other samples (crude juice, clarified sample and freeze-dried sample) showed low enzyme activity of less than 1500 GDU/g, with the activity decreasing significantly after freeze-drying. The proteolytic activity of bromelain seems to be related to its pH during gelatin digestion. The low pH of bromelain sample during the liberation of gelatin shows that the bromelain was proteolytically active with an increase of enzyme activity. Additional studies are needed to further investigate the effects of sample pH during gelatinization.

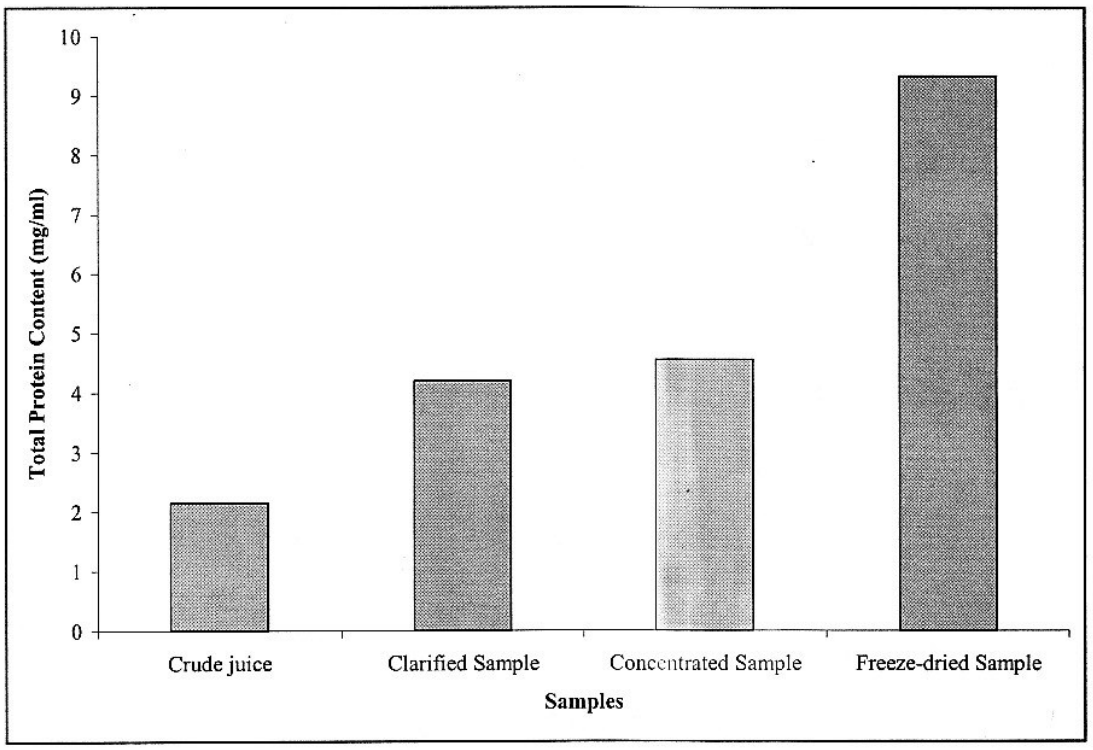

Fig. 4: Total protein content of crude, clarified, concentrated and freeze-dried samples of homogenized pineapple extract.

3.2 Effect of freeze-drying process on enzyme activity

Figure 5 shows significant changes in enzyme activity after freeze-drying process. At least 5-fold decrease in activity was observed at 1400 GDU/g of proteolytic activity after freeze-drying, which was determined during single-loop homogenization process. Although freeze-drying process did not decrease the protein content of bromelain extract, but its enzyme activity was significantly decreased. This shows that inactivation and instability of bromelain solution was detected. Thus, it is reasonable to hypothesize that different biologic effects may occur following exposure of bromelain solution to freeze-drying process. A reduction in enzyme activity was also observed for freeze-dried sample in non-homogenized extraction process, which decreased 5-folds from 8600 GDU/g (concentrated sample) to 1600 GDU/g after freeze-drying process [data not shown]. Thus, this validates that freeze-drying process decreased the enzyme activity of bromelain. Previous study suggested that concentrated bromelain solutions (>50 mg/ml) are more

resistant to spontaneous inactivation of their enzyme activity than are dilute solutions [9]. The sensitivity of bromelain protenaises to heat inactivation and their stability with respect to freezing suggests that fresh pineapple would be required to obtain sufficient benefits from proteolytic enzymes. However, fresh pineapple is not a concentrated source of bromelain enzymes. Therefore, our finding corresponds to the previous study carried out. Freeze-drying process is not recommended for diluted bromelain storage, especially bromelain from fresh pineapple. Additional studies are needed to be carried out to further assess the effects of freeze-drying process on the bromelain activity and stability, in order to overcome the sensitivity of bromelain enzymes in fresh pineapple and optimize bromelain proteolytic activity for commercial bromelain-tablets formulation.

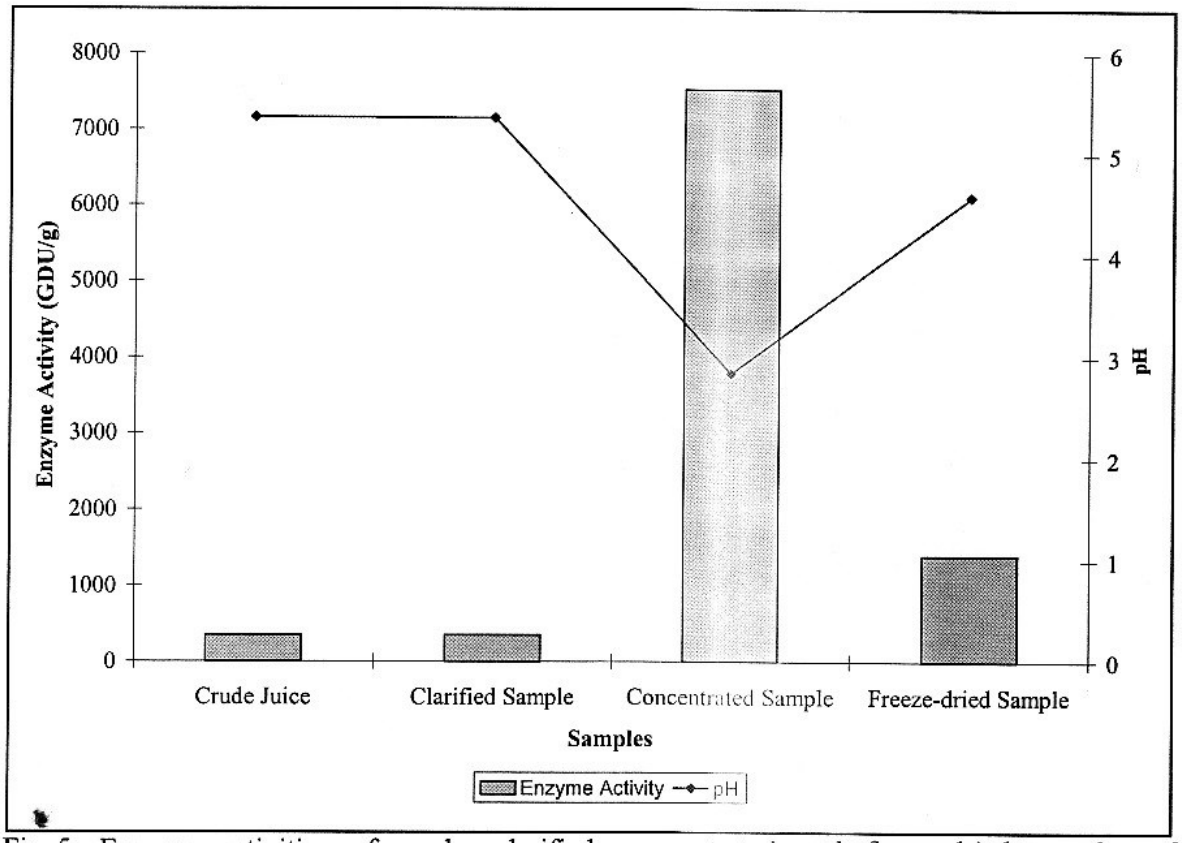

Fig. 5: Enzyme activities of crude, clarified, concentrated and freeze-dried samples of homogenized pineapple extract. Relationship and relevancy of enzyme activities and pH of each sample is presented.

4. Conclusion

The study reported here provides a preparation of fruit bromelain through extraction and homogenization that can produce an extract of core bromelain from fresh pineapple wastes. The data shown here exhibit relatively high protein content of 9.33 mg protein/ml and proteolytic activity of 1400 GDU/g in the core bromelain from fresh pineapple wastes obtained from the single-loop homogenization process. This study also shows the effects of homogenization process on total protein yield and proteolytic activity. Homogenization process is able to disrupt pineapple cells and release its proteolytic enzymes. A triple-loop homogenization produced an increase of protein yield to 12.13 mg/ml, and the enzyme activity was also increased by 3.6-fold when compared to single-loop homogenization. Freeze-drying process was found to inactivate bromelain enzymes and lower the proteolytic activity.

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